Influence of the Leader protein coding region of foot-and-mouth disease virus on virus replication

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INTRODUCTION

The foot-and-mouth disease virus (FMDV) positive-sense RNA genome (ca. 8400 nt) includes a single, long, open reading frame (ca. 7000 nt) encoding a polyprotein that is proteolytically processed to generate some 12 mature proteins plus multiple precursors (see Belsham, 2005). Members of the genus Aphthovirus, including FMDV, within the picornavirus family are unusual in having a protease as the first component of the polyprotein. The FMDV Leader (L) protein is a papain-like cysteine protease. It is produced in two distinct forms, termed Lab and Lb, resulting from initiation of protein synthesis at one of two initiation codons, usually 84 nt apart. Thus, the Lab protein has an N-terminal extension, which is highly variable (Carrillo et al., 2005), of 28 amino acids compared to Lb. Both the Lab and Lb forms of the protein can cleave the junction between the L protease and the structural protein precursor (P1-2A), and they also induce the cleavage of the translation initiation factor eIF4G (Medina et al., 1993). Indeed, both forms of this protein, eIF4GI and eIF4GII (46% identical), are cleaved in the presence of the FMDV L protease, albeit at distinct sites (Gradi et al., 2004; Kirchwegner et al., 1994). Loss of eIF4G can also occur in FMDV infected cells in the absence of the L protease since the FMDV 3C protease (3C^pro) also induces cleavage of this protein, albeit more slowly and at a different site (Belsham et al., 2000; Strong & Belsham, 2004). The loss of intact eIF4G results in the inhibition of cellular cap-dependent protein synthesis, but the initiation of protein synthesis on the FMDV RNA is maintained since it is dependent on an internal ribosome entry site (IRES) within the 5'-untranslated region (Belsham, 2005; Belsham & Brangwyn, 1990). The IRES-dependent translation of the uncapped FMDV RNA does not require the N-terminal region of eIF4G, including its associated eIF4E (the cap-binding protein).

The presence of the two separate sites for the initiation of protein synthesis on the FMDV RNA is conserved, for some unknown reason, in all natural strains of the virus (Carrillo et al., 2005; Sangar et al., 1987). The utilization of the two initiation sites varies between strains (Sangar et al., 1987). It seems that the FMDV IRES directs the translation initiation machinery to a site just upstream of the first functional initiation codon (Belsham, 1992; Ohlmann & Jackson, 1999) as with encephalomyocarditis virus (EMCV), a cardiovirus (see Kaminski et al., 1990, 1994) that shares with FMDV a similar type of IRES. However, many ribosomes fail to initiate protein synthesis at this point on the FMDV RNA and are then believed to scan along the RNA to the second initiation site (Belsham, 1992). Utilization of the two start sites is independent (Andreev et al., 2007; López de Quinto & Martínez-Salas, 1999). It has also been suggested that a minority of ribosomes may reach the second initiation site by an alternative mechanism (Pöyry et al., 2001).

The presence of the FMDV Lb coding sequence is not essential for virus viability within BHK cells in culture (Piccone et al., 1995). In contrast, deletion of the entire Lab
coding sequence is not tolerated. In addition, if the Lab start site is mutated (allowing only the Lb to be made) then viable viruses can still be produced; however, when the Lb start site was changed (permitting only the Lab form to be made) then no viable virus was obtained (Cao et al., 1995). These results could indicate that the region of the genome between the two start sites, termed the ‘spacer’ region, is required for some activity unrelated to its coding function.

Mutant FMD viruses (of strain A12) lacking the Lb coding sequence (termed A12LLV2) are attenuated in cattle and swine (Brown et al., 1996). The loss of the Lb coding sequence results in a failure of the mutant virus to block interferon induction (de los Santos et al., 2006). This can result from at least two separate effects: firstly the mutant viruses are less efficient at shutting down host cell protein synthesis (as described above), but secondly there is also a loss of the ability to degrade nuclear factor κB (NF-κB) (de los Santos et al., 2007).

In the studies of Piccone et al. (1995), deletion of the Lb coding sequence from the FMDV A12 cDNA was performed in a manner that left the two initiation codons intact within the mutant A12LLV2 virus. The same strategy was used in the recent studies by Uddowla et al. (2012) using the A24 strain of virus. In principle, these mutant viruses should produce two forms of the P1-2A capsid precursor. Initiation at the Lb start site should produce the authentic capsid precursor; however, initiation of translation at the Lab start site will result in an N-terminal extension to the P1-2A protein resulting in a product that cannot be modified by the cellular myristoylation system (Towler et al., 1988). Loss of myristoylation results in defective picornavirus capsid assembly or stability (Abrams et al., 1995; Belsham et al., 1991; Chow et al., 1987). Thus, the presence of the Lab start site, in addition to the Lab start site, could compromise the viability of the mutant FMD viruses lacking Lb alone. However, it is clear that this is not a total block since viruses of this form were obtained (Piccone et al., 1995; Uddowla et al., 2012). The A12LLV2 virus grows much more slowly than the wt A12 virus and an extended form of the VP0 protein (produced by 3Cpro mediated processing of the P1-2A precursor) from the LLV2 derivative of the A12 virus was apparent in addition to the authentic VP0 product (see Fig. 2 of Piccone et al., 1995). In contrast, the A24LL derivative, lacking Lb, grew with similar kinetics to the parental A24 virus but the capsid proteins produced by these viruses were not compared (Uddowla et al., 2012).

Recent studies have shown that insertion of a 57 nt transposon or an epitope tag within this ‘spacer’ region of the A24 virus can be tolerated but resulted in attenuation, whilst a 51 nt deletion did not adversely affect the growth of the virus in cell culture or the virulence of the virus in cattle (Piccone et al., 2010, 2011).

The analysis of the role of the Leader protease coding sequences during replication of FMDV in cell culture has now been extended. In particular, the requirement for the ‘spacer’ region has been determined and the rapid selection of previously undetected mutations that block the inappropriate modification of the capsid proteins has been observed.

**RESULTS**

**Production of modified FMDVs**

Modifications to the full-length cDNAs corresponding to the O1 Kaufbeuren (B64) strain of FMDV were performed using plasmid pT7S3 (Ellard et al., 1999) as the starting material. The modifications made to the cDNA are indicated in Fig. 1 and included the precise deletion of the Lb coding sequence, or the Lab coding sequence or the ‘spacer’ region between the two functional initiation codons. The plasmids were linearized and RNA transcripts were prepared in vitro and introduced into BHK cells (see Methods). Consistent with earlier results using serotype A virus (Piccone et al., 1995), precise deletion of the Lb coding sequence alone resulted in the generation of a viable virus (Table 1), whereas deletion of the entire Lab coding sequence blocked virus viability. Interestingly, the sole deletion of the 84 nt ‘spacer’ region, which represents the difference between the Lab deletion and the Lb deletion, did not affect virus viability (Table 1). Thus, the combined deletion of the Lb coding sequence and the ‘spacer’ element is lethal but these two modifications individually do not prevent virus viability.

**Deletion of Lb coding sequence alone results in production of modified VP0**

To determine the properties of the mutant FMDV RNAs lacking part or all of the Lab coding sequence, derivatives
of the full-length cDNAs shown in Fig. 1 were prepared by deleting much of the P2 and P3 coding sequences (including those for 3Cpro) to produce the ΔApa derivatives (see Methods). The resultant plasmids were transfected into cells infected with the vaccinia virus vTF7-3 (Fuerst et al., 1986) that expresses the T7 RNA polymerase. Each of the plasmids expressed the FMDV P1-2A capsid precursor along with any Leader protein sequences as indicated (Fig. 1). To analyse the functionality of the Leader protein coding sequences present within these ΔApa plasmids, the status of eIF4GI was assessed within extracts from transfected cells by immunoblotting using anti-eIF4GI antibodies. When either Lab or Lb (or both) were produced from the construct then quantitative cleavage of eIF4GI (to produce a cleavage product, termed CP) was observed (Fig. 2). However, when the sequences encoding Lab and/or Lb were deleted then no cleavage of eIF4GI was achieved. Deletion of the Lab ‘spacer’ region alone did not block the eIF4G cleavage since a functional Lb protease is still produced.

The P1-2A capsid precursor (or its extended derivative) was produced from each of these plasmids when transfected alone and there was no apparent difference in the size (ca. 90 kDa) of the unprocessed capsid protein precursor P1-2A that is produced (Fig. 3). Processing of the P1-2A precursor will not occur using these plasmids as 3Cpro is not encoded within these ΔApa derivatives (this was necessary to avoid 3C pro induced cleavage of eIF4G; Belsham et al., 2000). Thus, to achieve processing of the P1-2A precursor, the test plasmids were also co-transfected with plasmid pSKRH3C (Belsham et al., 2000) that expresses the FMDV 3Cpro. When the P1-2A molecules were processed (generating VP0, VP3, VP1 and 2A; see Polacek et al., 2013), the O1KwtΔApa, O1KLabΔApa and O1KLabspacerΔApa constructs each generated the authentic VP0 product (Fig. 3a). In contrast, when pSKRH3C was co-expressed with the O1KLabΔApa construct, two forms of VP0 were produced (Fig. 3a). One product, translated from the Lb initiation codon, corresponded to the authentic VP0 (as expressed from the O1KwtΔApa construct), but the second product was larger, presumably due to utilization of the Lab start site that will add 28 amino acids (ca. 3 kDa) to the N-terminus of VP0. There is no mechanism to achieve the removal of this additional sequence from the VP0 protein and it will block the myristoylation of the protein (see Introduction). These results indicated that although the O1KLab construct generated a viable virus, it could be expected that the capsid protein VP4 (and its precursor VP0) exist in two forms as generated from the O1KLabΔApa plasmid (Fig. 3a) and this will likely have an adverse effect on the virus.

![Fig. 2.](image-url)  
Fig. 2. Transient expression assays to determine eIF4G cleavage induced by the wt and mutant FMDV cDNAs. The indicated plasmids were transfected into vTF7-3 infected BHK cells as described in Methods. After 20 h, cell extracts were prepared and analysed by SDS-PAGE and immunoblotting using anti-eIF4G antibodies. The intact eIF4G and the cleavage product (CP), generated by expression of a functional FMDV Leader protein (Lab or Lb), are marked.

### Table 1. Summary of the properties of wt and mutant FMDV plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>eIF4G cleavage*</th>
<th>Authentic P1-2A†</th>
<th>Rescue of virus in BHK cells‡</th>
<th>Growth in pBTV cells§</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT7S3 (wt O1K)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>O1KLab</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>O1Lb</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>O1Lab spacer</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>O1Lab AUGmut</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Determined using ΔApa derivative, see Figs 1 and 2.  
† Determined using ΔApa derivative, see Figs 1 and 3.  
‡ See Fig. 4(a).  
§ See Fig. 4(b).  
||*Both authentic and extended form of P1-2A generated (see Fig. 3).
Leader protein coding sequence affects FMDV replication

To determine the influence of the Lab initiation codon on the properties of the Lab virus, this initiation codon (ATG) was modified to TTG within the O1K Lab plasmid so that translation initiation on the viral RNA can only occur at the second (Lb) start site. The modified plasmid (O1KLabAUGmut) was linearized, as above, and RNA transcripts were generated and electroporated into BHK cells. Viable mutant virus was obtained (Table 1).

Using the same strategy as described above, a derivative of the O1KLabAUGmut plasmid was prepared using Apal. Transient expression of this plasmid (O1KLabAUGmutApa) with pSKRH3C demonstrated that only the authentic VP0 product was now produced (Fig. 3b). This result confirmed that the additional FMDV protein species observed from plasmid O1KLabAUGmut was indeed resulted from initiation at the Lab start site.

**Growth kinetics of the Lb and LabAUGmut viruses in cell culture**

The growth characteristics of the wt and the viable mutant O1K viruses in BHK cells were examined in parallel (see Fig. 4a, b) using a similar multiplicity of infection in each case. The input virus was removed after 30 min absorption (defined as t=0) and the virus present at 0 h, 2 h, 4 h, 6 h and 24 h was determined by virus titration (Fig. 4a). Similarly, the level of FMDV RNA present at each time point was determined using quantitative real time RT-PCR (qRT-PCR) assays (Fig. 4b). Each of the viruses grew with similar kinetics but it was noted that at 4 h post infection, the wt virus yield (Fig. 4a) and the level of wt RNA accumulated (Fig. 4b) was higher than for the mutants. In addition, it was observed that although the same level of infectious virus (as determined by TCID50) had been applied to the cells initially, this resulted in rather different levels of input RNA. At both 0 h and 2 h, the level of FMDV RNA present in the cells infected with the O1KLabspacer and O1KLab mutants was about 10-fold higher than in BHK cells infected with the wt virus (Fig. 4b), a situation that was reversed at 4 h. This suggests that the particle to TCID50 ratio for these mutant viruses may be higher than for the wt virus.

The O1Kwt, O1KLab and O1KLabAUGmut viruses were also used to infect primary bovine thyroid cells (pBTY) and the level of viral RNA produced was determined (Fig. 4c). The O1Kwt virus grew well in these cells, but in contrast, no replication of either of the viruses lacking Lb was observed (Fig. 4c). Furthermore, in accord with these results, FMDV antigen ELISA assays failed to show the production of FMDV proteins in the pBTY cells infected with the mutant viruses, whereas high levels of antigen were observed in the O1Kwt-infected cells (data not shown).

**Selection of Lab viruses containing frameshift mutants within the ‘spacer’ region**

Although there was not a major growth advantage of the O1KLabAUGmut virus compared to the O1KLab virus during a single step growth cycle in BHK cells (Fig. 4a), the stability of these mutant viruses was analysed following virus passage. The initial mutant virus stocks (generated following three growth cycles) were passaged a further five times by overnight growth in BHK cells. Viral RNA was isolated after these additional passages and cDNA corresponding to the region of the genome including the two initiation codons was amplified by RT-PCR and sequenced. No change in the consensus sequence was apparent with the O1KLabAUGmut virus after these five passages (Fig. 5). However, even after the first passage (now P4) of the O1KLab virus, a low level of sequence heterogeneity was apparent downstream of the first initiation site and after the five additional passages the consensus sequence then became unreadable within this region (see Fig. 5) likely due to overlap of different individual sequences. To separate the sequences, the PCR product derived from the O1KLab viral RNA isolated after the five additional passages was inserted into a plasmid vector and from individual bacterial colonies, plasmid DNA was isolated and sequenced. At this stage, nine out of 16 sequences examined had mutations within the 84 nt ‘spacer’ region downstream of the Lab initiation site. The mutations observed are indicated in Fig. 6. Each sequence retained the
two AUG initiation codons, but three distinct variants (labelled V1, V2 and V3) were observed. Interestingly, the different mutations were either insertions (V1 and V2) or deletion (V3) of a single nucleotide within the `spacer' region. The mutations therefore changed the reading frame downstream from the Lab initiation codon, but did not affect the proteins generated from translation starting at the Lb initiation codon, which are required for virus production. The V1 and V2 insertions result in termination of the translation product that initiates from the Lab translation start site. The deletion of a single nucleotide in V3 means that translation will continue past the Lb initiation codon but the predicted product (49 amino acids) will terminate within the coding sequence of VP4 (read in a different frame).

**DISCUSSION**

The presence of two different forms of the Leader protein in all strains of FMDV strongly suggests that it is advantageous for the virus to be able to produce both forms of this protease. The expression of the Leader protein suppresses host cell protein synthesis, and this is believed to play an important role in the anti-host defence mechanism of the virus; for example, it will block the translation of interferon within infected cells (de los Santos et al., 2006). However, since both forms of the Leader protein display this activity (Medina et al., 1993), it is not clear what advantage is conferred by producing the Lab form in addition to the Lb protease.

Earlier studies have shown that precise deletion of the Lb protease from the A12 and A24 strains of FMDV produced viable viruses that replicate within BHK cells (Piccone et al., 1995; Uddowla et al., 2012). In contrast, deletion of the entire Lab sequence was not tolerated. These results were confirmed here using the O1Kaufbeuren virus (see Table 1). Cao et al. (1995) also demonstrated that the 84 nt `spacer' region did not need to be translated since modification of the Lab start site produced a viable virus (which still made Lb), whereas modification of the Lb start site did not. These earlier results left open the possibility that the `spacer' region could have a role that is separate from its coding function (e.g. as an RNA element). The results presented here clearly demonstrate that the `spacer' region is also not required as an RNA sequence for virus viability.

There was relatively little difference in the growth rate of the O1Kalb virus compared to the O1Kwt virus in BHK cells (Fig. 4), which contrasts with the much reduced growth rate of the A12LLV virus (Piccone et al., 1995), but which is consistent with recent results from a similar virus derived from the A24 strain (Uddowla et al., 2012). In addition, it is shown that, in the context of the virus lacking Lb (O1Kalb), modification of the Lab AUG codon to UGG still produced a viable virus. It was anticipated that this modification might improve the growth of the O1Kalb virus due to the removal of aberrant translation products that result in a modified form of the capsid proteins (see Fig. 3), but no major effect on growth in BHK cells was observed (Fig. 4). However, multiple passages of the O1Kalb virus in cells resulted in the emergence of various mutant viruses in which single nucleotides were deleted from, or inserted within, the 84 nt `spacer' region (Figs 5 and 6) although the Lab initiation codon itself was preserved. In contrast, when the Lab initiation codon had been modified (as in the O1KalbAUGmut virus), to prevent translation initiation from this point, then the sequence of the `spacer' region was maintained during such
**Fig. 5.** Multiple passages of O1KΔLb lead to emergence of mutant ‘spacer’ region sequences. The rescued O1KΔLb and O1KΔLbAUGmut viruses were passaged 5 times in BHK cells and the region of the FMDV RNA including the Lab and Lb initiation codons from samples harvested at the beginning (P4) and end of these passages (P8) was amplified by RT-PCR and consensus DNA sequences were determined from the PCR products. The chromatograms are shown; note the generation of overlapping sequences downstream of the GTTTT sequence located 14–18 nt downstream of the Lab initiation codon in the O1KΔLb sequence (indicated by →).

(a) Lab

```
M N T T D C F T I A L V Q A I R E I K A L F L S R T G K M
```

WT ATG.AAT.ACA.ACT.GAC.TGT.TTT.ACC.ATC.GCT.TTG.GTA.CAG.GCT.ATC.AGA.GAG.ATT.AAA.GCA.CTT.TTT.CTA.TCA.GAC.ACA.GSG.AAA.ATG

(b) Lab

```
M N T T D C F T I A L V Q A I R E I K A L F L S R T G K M
```

V1 ATG.AAT.ACA.ACT.GAC.TGT.TTT.ACC.ATC.GCT.TTG.GTA.CAG.GCT.ATC.AGA.GAG.ATT.AAA.GCA.CTT.TTT.CTA.TCA.GAC.ACA.GSG.AAA.ATG

(c) Lab

```
M N T T D C F T I A L V Q A I R E I K A L F L S R T G K M
```

V2 ATG.AAT.ACA.ACT.GAC.TGT.TTT.ACC.ATC.GCT.TTG.GTA.CAG.GCT.ATC.AGA.GAG.ATT.AAA.GCA.CTT.TTT.CTA.TCA.GAC.ACA.GSG.AAA.ATG

(d) Lab

```
M N T T D C F T I A L V Q A I R E I K A L F L S R T G K M
```

V3 ATG.AAT.ACA.ACT.GAC.TGT.TTT.ACC.ATC.GCT.TTG.GTA.CAG.GCT.ATC.AGA.GAG.ATT.AAA.GCA.CTT.TTT.CTA.TCA.GAC.ACA.GSG.AAA.ATG

**Fig. 6.** Identification of frameshift mutations within the ‘spacer’ region that emerged during passage of the O1KΔLb mutant FMDV. The amplicon generated from the O1KΔLb mutant virus at P8 was inserted into a plasmid vector and the sequences of plasmids from individual colonies were determined. The wt sequence is shown in (a) and three distinct variants (labelled V1, V2 and V3) that were identified in nine of 16 plasmids examined are shown in (b), (c) and (d). The alternative amino acid sequence that is produced following the frameshift mutation is indicated in red whereas the native FMDV protein sequence is indicated in black.
passages (Fig. 5). The mutations that occurred within the O1KΔLb virus modified the reading frame and prevented additional amino acid residues (derived from the ‘spacer’ region coding sequence) being fused to the amino-terminus of the authentic capsid precursor. No simple transition or transversion events were detected that modified the Lab initiation codon or, alternatively, introduced a stop codon within the ‘spacer’ region, although it is not essential to retain the Lab initiation codon for virus viability since it could be modified, as in the O1KΔLaUGmut virus. There are no indications of modification of this region within the A12LLV2 or A24LL viruses (Piccone et al., 1995; Uddowla et al., 2012), but the utilization of the two initiation sites can vary between different virus strains and if there is a stronger bias towards the use of the second site then the selection pressure will be less. The results reported here are consistent with earlier studies, using reporter gene constructs, which indicated that the presence of frameshift mutations within the ‘spacer’ region did not affect utilization of the Lb initiation codon (López de Quinto & Martínez-Salas, 1999).

Each of the deletions or insertion of nucleotides in the ‘spacer’ region occurred within a short homopolymeric region, presumably as a result of an error by the RNA polymerase. Typically, such transcriptional errors within the ORF, during RNA replication, will be lethal, but it is also possible that they may enhance the utilization of alternative ORFs, in a different region frame, e.g. for the L* protein of Thielers’s murine encephalomyelitis virus (TMEV) (Kong & Roos, 1991; van Eyll & Michiels, 2002) and the alternative ORF (termed 2B*) within the 2B coding region of EMCV (Loughran et al., 2011). It is interesting that this alternative ORF within EMCV occurs just downstream of a conserved GGUUUUUU motif and the frameshift mutations identified within the FMDV variants V1 and V3 involved an error within a run of four or five Us (see Fig. 6). If the RNA polymerase makes an error within this conserved motif of EMCV then the alternative ORF within the 2B coding sequence could be accessed without any requirement for ribosomal frameshifting. Although this sort of transcriptional error may be relatively rare, the high levels of viral RNA produced within infected cells may allow a significant amount of the alternative product to be made. Use of such an alternative ORF does not require an AUG initiation codon if accessed due to an insertion/deletion within the RNA sequence or by ribosomal frameshifting, since translation initiation occurs upstream of this region.

The results discussed above clearly show that the 84 nt ‘spacer’ region does not have to be translated and can even be deleted (O1KΔLabspacer, Table 1). However, this deletion could not be combined with deletion of Lb since deletion of the entire Lab was lethal (see Table 1) as described previously for the A12 virus (Piccone et al., 1995). This combination of two modifications, which themselves are tolerated to produce a lethal phenotype, appears analogous to the production of ‘synthetic lethal’ mutations within various organisms, e.g. yeast. Why should this combination of tolerated deletions result in a lethal phenotype? The FMDV RNA has to be translated under two different sets of conditions within infected cells. When the virus initially infects the cell, the RNA has to function in competition with the host mRNAs using the intact cap-binding complex (eIF4F, including eIF4G, eIF4A and eIF4E). However, once the FMDV Leader protein has been expressed then eIF4G is cleaved very rapidly (Belsham et al., 2000) and then most of the host mRNAs (which are capped and bound by eIF4E) are no longer used by the cellular translation machinery. Deletion of the Lb coding sequence from the viral genome means that this RNA has to continue to be translated under conditions similar to those encountered at the beginning of the infection cycle. Perhaps under these conditions there is a more efficient utilization of the RNA for translation if the sequence immediately downstream of the Lab initiation codon is present. Hence, loss of this ‘spacer’ sequence, under conditions of maximal competition with host mRNAs, may make the RNA sufficiently inefficient that it proves lethal. In the O1KΔLabspacer virus, the presence of the Lb coding sequence ensures that the cellular translation machinery is rapidly modified (through loss of intact eIF4G) and under these conditions then perhaps the loss of optimal translation efficiency for the viral RNA can be tolerated (at least in cell culture). However, it is not entirely clear why there is loss of viability resulting from modification of the Lb initiation site alone (Cao et al., 1995) since this does not seem very different from deleting the ‘spacer’ region alone as each of these mutants relies on initiation at the Lab start site.

The precise deletion of the Lb coding sequence, in combination with the modification of the Lab AUG codon, generates a virus that can be used to assess directly the role of the Lb protein in pathogenesis (without any possible involvement of the inappropriate addition of sequences to the N-terminus of the P1-2A as observed with the A12LLV (see Piccone et al., 1995) as described above). However, the cell culture adapted parental virus (O1K B64) used for the studies presented here displays an attenuated phenotype in cattle and pigs (Botner et al., 2011; Lohse et al., 2012) and hence these modifications need to be made in a virus background that is pathogenic. The inability of the O1KΔLaUGmut virus to replicate in pBTY cells (Fig. 4) (cf. the wt O1K B64 strain) may suggest that viruses of this type (within a pathogenic backbone) will also be attenuated in animals. Clearly, assessing the role of the FMDV Leader protein coding sequences on virus replication depends on the nature of the host system being analysed. However, the approach of precise engineering of FMDV to remove the Lb coding sequence with the modification of the Lab initiation codon offers the possibility of developing safer vaccines. In the event of virus escape, such strains can be expected to grow poorly, if at all, outside the BHK cells used for vaccine production.
**METHODS**

**Construction of plasmids containing full-length mutant FMDV cDNAs.** The plasmid pT7S5 (Ellard et al., 1999) contains the full-length cDNA for the O1K B64 strain of FMDV. To modify the L coding region, a KpnI fragment (ca. 1.4 kb) from pT7S5 was inserted into KpnI-digested pGEM4Z (Promega) and then used as the template in overlap PCRs using the M13F primer with reverse primers (LabDelrev, LbDelrev and LspacerDelrev) and in separate reactions using the M13R primer with the forward primers (LabDelfor, LbDelfor and Lspacerfor); all primer sequences are shown in Table 2. The primary PCR products were gel purified, mixed appropriately, annealed and then used in further PCRs with just the M13F and M13R primers. The final products were gel purified and introduced into pCR-XL-TOPO (Invitrogen). The modified fragments were released with KpnI and constructed back into KpnI-digested pT7S5. The resultant plasmids were then sequenced to confirm the structures (see Fig. 1).

Modification of the Lab initiation codon in the ΔLab background was achieved by site-directed mutagenesis using PhTF (Agilent) using the primers LabAUG-UGGfor and LabAUG-UGGrev (Table 2) with the O1KLb cDNA in pCR-XL-TOPO (Invitrogen) as template. The mutant sequence was confirmed by sequencing and the modified KpnI fragment was reconstructed back into the full-length cDNA as described above.

The ΔApa derivatives of the wt and mutant full-length FMDV cDNAs were prepared by digesting the plasmids with Apal to liberate fragments of 2832 bp and 259 bp derived from the cDNA corresponding to the whole of the P2 and part of the P3 coding sequence (see Fig. 1). The large residual fragment was, in each case, gel purified, self-ligated and transformed into E. coli. Plasmids of the correct structure were amplified and checked by sequencing. The resultant plasmids can express, from the T7 promoter, the FMDV L (where applicable) and P1-2A coding regions only.

**Transient expression assays.** Plasmid DNAs were transfected, using FuGene6 (Roche), into BHK cells that had been infected with the vaccinia virus vTF7-3 (indicating no carryover of plasmid). The fragments (ca. 450 bp) were transfected, and control reactions, in the absence of RT, no products were obtained (indicating no carryover of plasmid). The transcripts (ca. 450 bp) were sequenced directly using the same primers, and when necessary the fragments were amplified again (Table 2).

**Table 2. Oligonucleotides used to create and sequence mutant FMDV cDNAs**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’–3’)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13F</td>
<td>GTAAACGACGCGCAT</td>
</tr>
<tr>
<td>M13R</td>
<td>CAGGAAACAGCATGAC</td>
</tr>
<tr>
<td>LabDelrev</td>
<td>GACTGATTGTCTCAGCCAGCCCCATGTTTCAGTGGTTTAAAG</td>
</tr>
<tr>
<td>LbDelrev</td>
<td>GACTGATTGTCTCAAGCGCCCCATCGTGGTTGCG</td>
</tr>
<tr>
<td>LspacerDelrev</td>
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<tr>
<td>LabDelfor</td>
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<tr>
<td>LbDelfor</td>
<td>GGCACACAGGGGAAAAATGGGGGGTGGGAAATCCGTCGT*</td>
</tr>
<tr>
<td>LspacerDelfor</td>
<td>CTTTTATACAACATGAAACGCTGGACAATCCGTCGTACCGG</td>
</tr>
<tr>
<td>LabAUG-UGGfor</td>
<td>ATAACCACCTGAAACTCAGAATAACAACGTGATCT</td>
</tr>
<tr>
<td>LabAUG-UGGrev</td>
<td>CATCAGTTGTATCCAGTGTCACTGTGGTTTAT</td>
</tr>
<tr>
<td>8-APN3</td>
<td>GGCTAAAGATGCCCCCTCAG</td>
</tr>
<tr>
<td>8-APN4</td>
<td>AACCAGCTXTTCTTXGTXGTG†</td>
</tr>
</tbody>
</table>

*The linkage of the FMDV initiation codons to the start of the capsid protein coding sequence is indicated in the bold, italic and underlined sequence. †X = inosine.
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